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Original Article

Postoperative Prognostic Predictors of Bile Duct Cancers: Clinical Analysis and Immunoassays of Tissue Microarrays

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Background/Aims: Cholangiocarcinoma frequently recurs even after curative resection. Expression levels of proteins such as epidermal growth factor receptor (EGFR), Snail, epithelial cadherin (E-cadherin), and interleukin-6 (IL-6) examined by immunohistochemistry have been studied as potential prognostic factors for cholangiocarcinoma. The aim of this study was to investigate significant factors affecting the prognosis of resectable cholangiocarcinoma.

Methods: Ninety-one patients who underwent surgical resection at Samsung Medical Center for cholangiocarcinoma from 1995 to 2013 were included in this study. Expression levels of E-cadherin, Snail, IL-6, membranous EGFR, and cytoplasmic EGFR were analyzed by immunohistochemistry using tissue microarray blocks made from surgical specimens.

Results: Patients with high levels of membranous EGFR in tissue microarrays had significantly shorter overall survival (OS) and disease-free survival (DFS): high membranous EGFR (score 0–2) 38.0 months versus low membranous EGFR (score 3) 14.4 months (p=0.008) and high membranous EGFR (score 0–2) 23.2 months versus low membranous EGFR (score 3) 6.1 months (p=0.004), respectively. On the other hand, E-cadherin, Snail, cytoplasmic EGFR, and IL-6 did not show significant association with OS or DFS. Patients with distant metastasis had significantly higher IL-6 levels than those with locoregional recurrence (p=0.01).

Conclusions: This study showed that overexpression of membranous EGFR was significantly associated with shorter OS and DFS in surgically resected bile duct cancer patients. In addition, higher IL-6 expression was a predictive marker for recurrence in cholangiocarcinoma patients with distant organ metastasis after surgical resection. **(Gut Liver 2023;17:159-169)**

Key Words: Cholangiocarcinoma; Immunohistochemistry; Microarray; Prognosis

INTRODUCTION

Cholangiocarcinoma (CCA) originating from the bile duct epithelial cells or cholangiocytes is an aggressive malignancy with a poor prognosis.[1-3] Surgical resection is the only curative and most effective treatment for CCA.[3-5] However, most patients with CCA are in an advanced stage at the time of presentation and the recurrence of CCA is high even in curatively resected patients.[3,4] In curatively resected patients, the 5-year overall survival (OS) rate is 20% to 40%.[3,6] Therefore, it is important to identify prognostic factors in patients with bile duct cancer after surgery.

Protein expression has been suggested as a prognostic factor or potential therapeutic target in various cancers. Some proteins are thought to play a role in tumor invasion, progression, recurrence and metastasis. Several studies have been done about the role of protein expression in tumor progression or invasion. It has been suggested that

high epidermal growth factor receptor (EGFR) expression is related to tumor progression and recurrence.[7,8] In previous studies, the expression of EGFR has been suggested as a negative predictor of the prognosis of bile duct cancer.[8-10] The inflammatory cytokine interleukin-6 (IL-6) enhances tumor growth by altered EGFR expression via EGFR promoter methylation in CCA.[11] IL-6 triggers epithelial-mesenchymal transition (EMT) in CCA cells by promoting downregulation of epithelial cadherin (E-cadherin).[12,13] Low expression of E-cadherin is associated with tumor recurrence and poor OS.[13-20] Bile acids repress E-cadherin through the induction of EMT-inducing transcription factor Snail and increase cancer invasiveness in human CCA.[21] High expression of Snail is associated with lymph node metastasis and poor survival in CCA.[22]

In this study, we evaluated immunohistochemical scores of the following proteins on tissue microarrays (TMA) of R0 or R1 resected CCA known to contribute to disease progression: E-cadherin, Snail, IL-6, membranous EGFR (EGFR-M), and cytoplasmic EGFR (EGFR-C).

MATERIALS AND METHODS

1. Study patients and clinical data

Patients who underwent surgical resection of CCA with curative intent were included in this study at Samsung Medical Center, Seoul, Korea. This study was conducted in accordance with the Declaration of Helsinki. It was approved by the Institutional Review Board of Samsung Medical Center, Seoul, Korea (IRB number: SMC 2015-05-044-006). Medical records of patients enrolled in this study were reviewed using an electronic record system of Samsung Medical Center. Written informed consents were renounced because of the retrospective nature of the study. The following data were reviewed: age, sex, type of CCA (intrahepatic, perihilar, or distal CCA), pathologic stage (the American Joint Committee on Cancer [AJCC] 8th edition), histologic differentiation and laboratory data.

Clinical outcomes were OS and disease-free survival (DFS). OS was defined as the length of time after operation until death by any cause. DFS was defined as the length of time after operation until the first progression or death by any cause, if disease progression did not occur based on radiographic imaging studies. Disease recurrence was evaluated by standardized radiographic imaging studies.

2. Immunohistochemistry

Core tissue biopsies of 2 mm in thickness were extracted from individual formalin fixed and paraffin embedded CCAs (donor blocks). In each case, two representative cores were constructed and incorporated into recipient paraffin blocks of TMA. Four micrometer sections were cut from TMA blocks to generate TMA slides for immunohistochemical analyses.

Immunohistochemistry (IHC) was performed to evaluate expression levels of E-cadherin, EGFR, Snail, and IL-6. Primary antibodies and their dilution used for IHC were E-cadherin (1:200, cat# M3612; DAKO, Glostrup, Denmark), EGFR (1:100, cat# NCL-L_EGFR-384; Leica Biosystems, Nussloch, Germany), IL-6 (1:400, cat# ab6672; Abcam Inc., Eugene, OR, USA) and Snail (1:100, cat# NBP2-32768; Novus Biologicals, Littleton, CO, USA). E-cadherin, EGFR and IL-6 IHC studies were performed using a BOND-MAX automated stainer (Leica Biosystems) according to the manufacturer's instructions. Snail immunostaining was done using an OptiView DAB IHC Detection Kit (Ventana Medical Systems, Tucson, AZ, USA). Immunoexpression of each protein was scored based on the proportion of stained cells: 0 for negligible staining (<10%), 1 for focal staining (10% to 25%), 2 for substantial staining (25% to 50%) and 3 for diffuse staining (>50%). The scoring of IHC was performed independently by two pathologists (Y.K. and K.T.J.). If there were differences between the two, slides were re-evaluated jointly by both investigators and finally decided.

3. Statistical analysis

Nonparametric tests were used. Survival curves were evaluated with the Kaplan-Meier method. The log-rank test was done to evaluate the significance of differences between survival curves. The Cox proportional hazard regression modeling was performed for multivariate analysis. We used additive models to confirm whether each IHC marker was a significant prognostic factor. And multivariate analysis was performed about clinicopathologic variables, and a reference model was constructed with independent factors with statistical significance by using backward variable selection. Next, the significance of each marker was verified through the process of performing multivariate analysis by adding each marker to the reference model one by one. The Fisher exact test was used to evaluate whether the expression rate of each IHC marker differed between those with distant organ metastasis and those with locoregional recurrence among relapsed patients. The sample was divided into subgroups of <3 years, 3–5 years, and >5 years according to the length of OS. Linear by linear association was used to analyze whether the expression of each IHC marker differed according to the survival time. The Mann-Whitney test and the Kruskal-Wallis test were used to evaluate the correlation between each IHC marker and location of tumor and morphology. Statistical significance was set at a p-value <0.05. Kaplan-

Meier method, Fisher exact test and linear by linear assessment were performed using IBM SPSS statistics version 28 (IBM Corp., Armonk, NY, USA). The Cox proportional hazard regression modeling was performed using software R (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

1. Patient characteristics

A total of 91 patients who underwent surgical resection of CCA with curative intentions were included in this study. Clinicopathologic characteristics and immunohistochemical staining results of the 91 patients are summarized in Table 1. The median age of patients at the time of diagnosis was 65 years. There were 65 males (71%) and 26 females (29%). Tumors were located at the intrahepatic area in 22 (24%), perihilar in 34 (37%), and distal in 35 (39%) patients. Based on AJCC 8th edition, 17 patients (19%) had stage I disease, 52 (59%) had stage II disease and 22 (24%) had stage III disease. CCA was classified into well differentiated in 18 patients (20%), moderately differentiated in 46 (50%), and poorly differentiated in 27 (30%). According to pathology after surgery, 74 cases (81%) had R0 resection and 17 cases (19%) had R1 resection. *Clonorchis sinensis* infection was defined as positive based on results of enzyme-linked immunosorbent assay, stool test, and skin test or as confirmed in surgical specimen or bile. It was found that 22% of patients were positive for *C. sinensis* infection. The median serum level of carbohydrate antigen 19-9 (CA19-9) was 73.51 U/mL and that of carcinoembryonic antigen (CEA) was 2.10 ng/mL.

2. Reference model of OS according to clinicopathologic factors

The median OS of total patients was 31.0 months. Table 2 shows the results of univariate and multivariate analyses of clinicopathologic factors. The following factors were statistically significant predictors of OS in univariate analysis: age, AJCC 8th stage, resection margin, pathologic differentiation, CEA, CA19-9, and alkaline phosphatase. Multivariate analysis was performed using variables with p-value <0.1 in univariate analysis to make a reference model based on clinicopathologic factors. The location of tumor is an important variable. It is known as a factor affecting the prognosis.[5] Thus, it was included in the multivariate analysis regardless of the p-value. In multivariate analysis, AJCC 8th stage was identified as a significant factor affecting the OS (p<0.001). Thus, it was included in the reference model. Finally, the reference model for OS included tumor staging and location of tumor.

Table 1. Clinicopathologic Characteristics of the Patients and Immunohistochemical Markers in Tissue Microarrays

Variable		Value (n=91)
Age, yr		65 (37–89)
Sex	Male	65 (71)
	Female	26 (29)
Tumor location	Intrahepatic CCA	22 (24)
	Perihilar CCA	34 (37)
	Distal CCA	35 (39)
Morphology	Periductal infiltrating type	52 (57)
	Mass forming type	22 (24)
	Intraductal growing type	17 (19)
Tumor stage (AJCC 8th)	I	17 (19)
	II	52 (57)
	III	22 (24)
Resection margin	R0	74 (81)
	R1	17 (19)
Differentiation	Well	18 (20)
	Moderate	46 (50)
	Poor	27 (30)
Diabetes mellitus	No	79 (87)
	Yes	12 (13)
Smoking*	No	27 (30)
	Yes	62 (70)
Clonorchis sinensis infection	No	71 (78)
	Yes	20 (22)
BMI, kg/m^2		22.82 (16.59–31.71)
CEA, ng/mL		2.10 (0.50–55.95)
CA19-9, U/mL		74 (4–10,530)
Total bilirubin, mg/dL		2.5 (0.2–37.9)
ALP, U/L		223 (54–1,390)
IHC marker		
E-cadherin	0	3 (3)
	1–3	88 (97)
Snail	0	26 (28)
	1–3	65 (72)
IL-6	0	39 (43)
	1–3	52 (57)
EGFR-M	0–2	71 (78)
	3	20 (22)
EGFR-C	0–2	46 (51)
	3	45 (49)

Data are presented as median (interquartile range) or number (%).
CCA, cholangiocarcinoma; AJCC, American Joint Committee on Cancer; BMI, body mass index; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; ALP, alkaline phosphatase; IHC, immunohistochemistry; E-cadherin, epithelial cadherin; IL-6, interleukin-6; EGFR-M; membranous epidermal growth factor receptor, EGFR-C; cytoplasmic epidermal growth factor receptor.
*Loss (n=2).

Table 2. Univariable and Multivariable Analyses of Clinicopathologic Factors and Immunohistochemical Markers for Overall Survival

Predictor	Univariable model		Multivariable model	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Sex (male vs female)	1.12 (0.66–2.74)	0.678		
Age (median: <65 vs ≥65 yr)	1.69 (1.04–2.74)	0.033		
Intrahepatic & perihilar vs distal CCA	1.38 (0.85–2.24)	0.191	1.51 (0.91–2.52)	0.112
Morphology		0.108		
Periductal infiltrating	Reference			
Mass forming	1.15 (0.66–2.00)	0.622		
Intraductal growing	0.51 (0.26–1.03)	0.061		
Tumor staging		<0.001		<0.001
I	Reference		Reference	
II	5.32 (2.19–12.9)	<0.001	5.06 (2.08–12.32)	<0.001
III	9.15 (3.53–23.7)	<0.001	9.92 (3.80–25.93)	<0.001
Resection margin (R0 vs R1)	1.77 (1.01–3.12)	0.046		
Differentiation		0.001		
Well differentiated	Reference			
Moderately differentiated	3.16 (1.47–6.81)	0.003		
Poorly differentiated	4.79 (2.10–10.90)	<0.001		
Diabetes mellitus (no vs yes)	0.66 (0.30–1.45)	0.301		
Clonorchis sinensis infection (no vs yes)	0.59 (0.32–1.10)	0.095		
BMI (median: <22.81 vs ≥22.81 kg/m^2)	0.93 (0.58–1.50)	0.772		
CEA (UNL: <5 vs ≥5 ng/mL)	2.02 (1.02–4.02)	0.044		
CA19-9 (UNL: <37 vs ≥37 U/mL)	1.93 (1.12–3.30)	0.017		
Total bilirubin (median: <2.5 vs ≥2.5 mg/dL)	1.52 (0.93–2.48)	0.096		
ALP (UNLx1.5: <150 vs ≥150 U/L)	1.86 (1.07–3.22)	0.028		
IHC marker				
E-cadherin (0 vs 1–3)	2.92 (0.41–21.1)	0.287	4.73 (0.64–34.97)	0.128
Snail (0 vs 1–3)	1.32 (0.75–2.31)	0.340	1.13 (0.63–2.00)	0.685
IL-6 (0 vs 1–3)	1.25 (0.77–2.02)	0.364	0.95 (0.58–1.55)	0.832
EGFR-M (0–2 vs 3)	2.17 (1.24–3.78)	0.006	2.22 (1.27–3.90)	0.005
EGFR-C (0–2 vs 3)	1.12 (0.70–1.79)	0.646	1.22 (0.76–0.96)	0.416

HR, hazard ratio; CI, confidence interval; CCA, cholangiocarcinoma; BMI, body mass index; CEA, carcinoembryonic antigen; UNL, upper normal limit; CA19-9, carbohydrate antigen 19-9; ALP, alkaline phosphatase; IHC, immunohistochemistry; E-cadherin, epithelial cadherin; IL-6, interleukin-6; EGFR-M, membranous epidermal growth factor receptor; EGFR-C, cytoplasmic epidermal growth factor receptor.

3. Evaluation of Immunohistochemical profiles of each marker with OS in CCA

We performed immunohistochemical staining to analyze five different markers in CCA. The expression of E-cadherin, Snail, IL-6, and EGFR was assessed with CCA TMA, and its level of each marker was scored from 0 to 3 depending on its proportion of stained cells (Fig. 1). The positivity of each marker varied from 37% to 97% on TMAs. EGFR was immunohistochemically stained in cytoplasm (EGFR-C) and membrane (EGFR-M). It was expressed in only cytoplasm of 37 CCA tissues, and in both cytoplasm and membrane of 34 CCA tissues with different scores (Fig. 2A, Supplementary Table 1).

The relationship between each IHC scores and OS are presented in Table 2 and Supplementary Table 2. Univariate analysis revealed that patients with high expression of EGFR-M had significantly shorter survival. Also, E-cadherin, Snail, IL-6, and EGFR-C did not show any significant relationship with OS in the univariate analysis. The additive model was used to determine if each marker was a significant factor for OS. Multivariable analysis was performed by adding each IHC marker to the reference model based on the clinicopathologic factor. In multivariate analysis, patients with high expression of EGFR-M had significantly shorter survival (hazard ratio, 2.22; 95% confidence interval, 1.27 to 3.90; p=0.005). In the Kaplan-Meier analysis, lower expression of EGFR-M was significantly related to longer survival. The median OS was 38.0 months for low EGFR-M (score 0–2) and 14.4 months for high EGFR-M (score 3) (p=0.008) (Fig. 2B).

When we divided patients into three groups depending on OS length, 49, 10, and 32 patients showed OS within 3 years, from 3 to 5 years, and above 5 years, respectively. Among patients with OS <3 years, 31% showed high EGFR-M expression, whereas in patients with OS >5 years, only 9% showed high EGFR-M expression. There was a significant relationship between the high expression of EGFR-M and the length of OS (p=0.024) (Table 3, Supplementary Table 3).

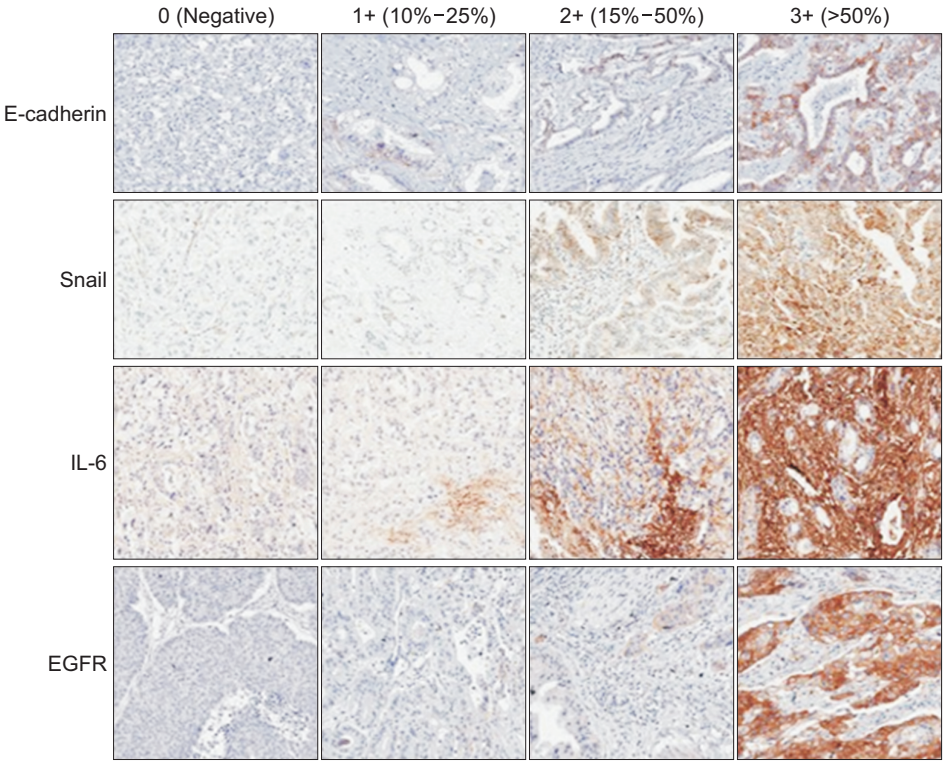

Fig. 1. Immunohistochemical (IHC) staining of cholangiocarcinoma (CCA). IHC staining of epithelial cadherin (E-cadherin), Snail, interleukin-6 (IL-6), and epidermal growth factor receptor (EGFR) protein expression in CCA tissues (×400). The IHC scoring of bile duct cancer was rated from 0 to 3, and the positivity varied from 1% to 10%.

4. Reference model of DFS according to clinicopathologic factors

The median DFS of total patients was 20.2 months. Results of univariate and multivariate analyses of clinicopathologic factors for DFS are shown in Table 4. The following variables were statistically significant predictors of OS in univariate analysis: AJCC 8th stage, pathologic differentiation, and CEA. Multivariate analysis was performed including variables with p-value <0.1 in univariate analysis to make a reference model. The location of tumor was also included in the multivariate analysis and the final reference model. In multivariate analysis, differentiation was identified as a significant factor affecting the DFS (p=0.003). Therefore, the final reference model for DFS included pathologic differentiation and tumor location.

5. Assessment of Immunohistochemical profiles of each marker with DFS in CCA

Analysis results for IHC markers and DFS are shown in Table 4 and Supplementary Table 4. In univariate analysis, patients with high expression of EGFR-M had significantly shorter DFS. The expression of E-cadherin, Snail, IL-6, and EGFR-C did not affect DFS in the univariate analysis. In multivariate analysis performed by adding the reference model, patients with high expression of EGFR-M had significantly shorter DFS (hazard ratio, 2.30; 95% confidence interval, 1.16 to 4.55; p=0.017). In the Kaplan-Meier analysis, the median DFS was 23.2 months for low EGFR-M (score 0–2) versus 6.1 months for high EGFR-M (score 3) groups (p=0.004) (Fig. 2C).

6. Analysis of Immunohistochemical profiles upon Distant organ metastasis, Tumor location and Morphology

The expression of IHC markers was analyzed to identify predictors affecting patterns of metastases (locoregional vs distant) (Table 5, Supplementary Table 5). The Fisher exact test was used to evaluate whether the percentage of expression of each IHC marker differed between patients with distant organ metastasis and those with locoregional recurrence. Patients with distant metastases had significantly higher expression of IL-6 than those with locoregional recurrences (p=0.010). When analyzing the relationship between each marker (E-cadherin, Snail, IL-6, EGFR-M, and EGFR-C) and the location of tumor and morphology, there was no statistical correlation (Supplementary Tables 6 and 7).

DISCUSSION

CCA has unfavorable outcome due to its late diagnosis and poor response to therapy.[4,23] CCA can also easily recur or metastasize, even in patients with curative resection.[24]

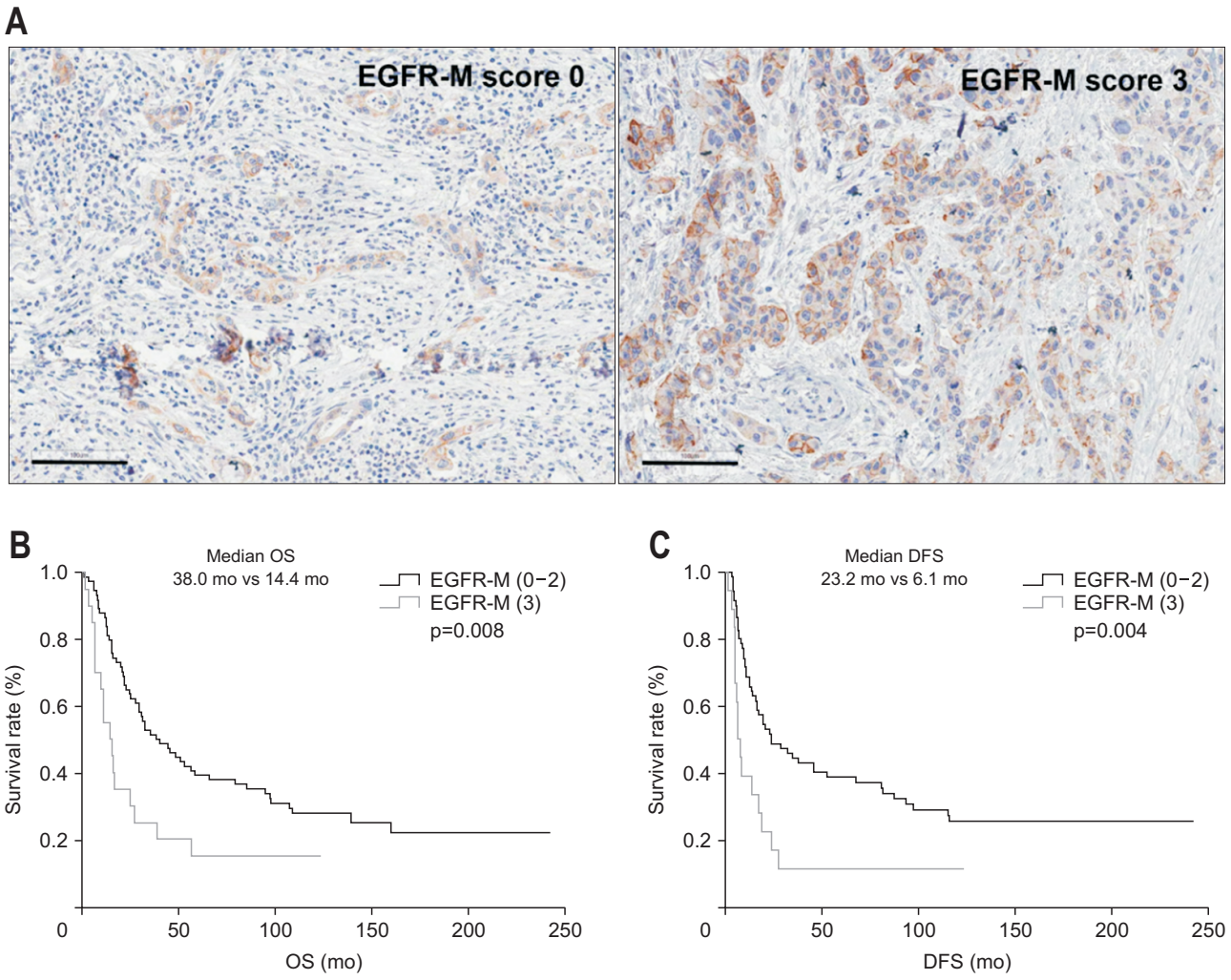

Fig. 2. Kaplan-Meier survival curves for overall survival (OS) and disease-free survival (DFS) according to the expression of membranous epidermal growth factor receptor (EGFR-M). (A) Representative images of EGFR-M expression (scale bar=100 μm). Kaplan-Meier analysis of EGFR-M expression with OS (B) and DFS (C).

Table 3. Association between the Length of Overall Survival and Immunohistochemical Markers

IHC marker		Overall survival, No. (%)			p-value
		<3 yr (n=49)	3–5 yr (n=10)	>5 yr (n=32)	
E-cadherin	0	1 (2)	0	2 (6)	0.325
	1–3	48 (98)	10 (100)	30 (94)	
Snail	0	15 (31)	1 (10)	10 (31)	0.972
	1–3	34 (69)	9 (90)	22 (69)	
IL-6	0	19 (39)	5 (50)	15 (47)	0.454
	1–3	30 (61)	5 (50)	17 (53)	
EGFR-M	0–2	34 (69)	8 (80)	29 (91)	0.024
	3	15 (31)	2 (20)	3 (9)	
EGFR-C	0–2	25 (51)	4 (40)	17 (53)	0.894
	3	24 (49)	6 (60)	15 (47)	

IHC, immunohistochemistry; E-cadherin, epithelial cadherin; IL-6, interleukin-6; EGFR-M, membranous epidermal growth factor receptor; EGFR-C, cytoplasmic epidermal growth factor receptor.

Clinicopathologically, the prognosis after surgical resection in CCA patients is affected by factors such as tumor location, TNM stage, histological differentiation, resection margin, CEA, and CA19-9.[25] These traditional prognostic factors are insufficient to predict disease recurrence, distant organ metastases and OS in patients with CCAs. Several mechanisms of metastasis or recurrence after resection have been proposed and multiple molecular expressions are thought to affect cancer recurrence and metastasis. By identifying molecular markers related to the progression of CCAs, high-risk groups after therapeutic resection can be distinguished.

In our study, the median OS and DFS in patients who showed overexpression of EGFR-M were significantly shorter than those of others. This suggests that EGFR-M overexpression is a negative predictor of CCA. On the other hand, EGFR-C overexpression did not show difference

Table 4. Univariable and Multivariable Analyses of Clinicopathologic Factors and Immunohistochemical Markers for Disease-Free Survival

Predictor	Univariable model		Multivariable model	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Sex (male vs female)	1.07 (0.62–1.83)	0.811		
Age (median: <65 vs ≥65 yr)	1.48 (0.90–2.43)	0.126		
Intrahepatic & perihilar vs distal CCA	1.38 (0.83–2.28)	0.210	1.32 (0.75–2.32)	0.341
Morphology		0.069		
Periductal infiltrating	Reference	-		
Mass forming	1.32 (0.74–2.35)	0.344		
Intraductal growing	0.51 (0.24–1.05)	0.069		
Tumor staging		0.002		
I	Reference	-		
II	2.60 (1.25–5.43)	0.011		
III	4.28 (1.88–9.72)	<0.001		
Resection margin (R0 vs R1)	1.42 (0.77–2.62)	0.265		
Differentiation		0.003		0.003
Well differentiated	Reference	-	Reference	-
Moderately differentiated	3.12 (1.44–6.77)	0.004	3.87 (1.65–9.92)	0.002
Poorly differentiated	4.15 (1.80–9.57)	0.001	4.94 (1.94–12.60)	0.001
Diabetes mellitus (no vs yes)	0.98 (0.47–2.06)	0.957		
Clonorchis sinensis infection (no vs yes)	0.93 (0.52–1.66)	0.804		
BMI (median: <22.81 vs ≥22.81 kg/m^2)	0.93 (0.57–1.53)	0.781		
CEA (UNL: <5 vs ≥5 ng/mL)	2.50 (1.25–5.01)	0.010		
CA19-9 (UNL: <37 vs ≥37 U/mL)	1.44 (0.84–2.47)	0.181		
Total bilirubin (median: <2.5 vs ≥2.5 mg/dL)	1.13 (0.69–1.87)	0.626		
ALP (UNLx1.5: <150 vs ≥150 U/L)	1.33 (0.77–2.29)	0.299		
IHC markers				
E-cadherin (0 vs 1–3)	3.14 (0.44–22.70)	0.256	8.15 (1.07–62.28)	0.043
Snail (0 vs 1–3)	1.16 (0.65–2.09)	0.615	0.81 (0.43–1.52)	0.511
IL-6 (0 vs 1–3)	1.19 (0.72–1.97)	0.497	0.87 (0.51–1.46)	0.589
EGFR-M (0–2 vs 3)	2.44 (1.37–4.33)	0.003	2.30 (1.16–4.55)	0.017
EGFR-C (0–2 vs 3)	1.06 (0.65–1.74)	0.811	0.91 (0.55–1.51)	0.703

HR, hazard ratio; CI, confidence interval; CCA, cholangiocarcinoma; BMI, body mass index; CEA, carcinoembryonic antigen; UNL, upper normal limit; CA19-9, carbohydrate antigen 19-9; ALP, alkaline phosphatase; IHC, immunohistochemistry; E-cadherin, epithelial cadherin; IL-6, interleukin-6; EGFR-M, membranous epidermal growth factor receptor; EGFR-C, cytoplasmic epidermal growth factor receptor.

Table 5. Evaluation of Immunohistochemical Markers and Distant Organ Metastasis

IHC marker		No. (%)		p-value
		Locoregional recurrence (n=16)	Distant metastasis (n=44)	
E-cadherin	0	0	1 (2)	1.000
	1–3	16 (100)	43 (98)	
Snail	0	1 (6)	13 (29)	0.086
	1–3	15 (94)	31 (71)	
IL-6	0	11 (69)	14 (42)	0.010
	1–3	5 (31)	30 (58)	
EGFR-M	0–2	13 (81)	32 (73)	0.738
	3	3 (19)	12 (27)	
EGFR-C	0–2	9 (56)	22 (50)	0.668
	3	7 (44)	22 (50)	

IHC, immunohistochemistry; E-cadherin, epithelial cadherin; IL-6, interleukin-6; EGFR-M, membranous epidermal growth factor receptor; EGFR-C, cytoplasmic epidermal growth factor receptor.

in prognosis (Tables 2 and 4, Fig. 2B and C).

EGFR overexpression has been reported to play an important role in increased tumor invasion and metastasis of various cancers.[23] EGF can induce cell detachment from the extracellular matrix and increase cell motility in patients with EGFR overexpression.[26] This tendency has been reported in multiple cancers including lung and breast cancers.[27] In previous studies, EGFR mutations have been observed in 10% to 15% of CCA, like other cancers.[28-30]

Cellular localization pattern of EGFR has been studied as a prognostic and predictive marker in many other cancers. However, the role of subcellular localization of EGFR on tumor prognosis has shown heterogeneous results. Pu *et al.*[31] have demonstrated that EGFR-M staining is significantly stronger in renal cell carcinoma tumors, whereas EGFR-C staining is significantly higher in normal tissues. They suggested that different locations of EGFR expression

might be associated with human renal tumorigenesis. On the other hand, Kallio *et al.*[32] have shown that OS of renal cell carcinoma patients with prominent EGFR-M staining was significantly longer than patients with mainly EGFR-C staining. In the study by Mahipal *et al.*,[33] only EGFR-M overexpression in pancreatic cancer patients was associated with worse clinical outcomes, whereas, in the study by Ueda *et al.*,[34] cytoplasmic overexpression of EGFR plays a significant role in the progression of pancreatic ductal adenocarcinoma. Also, overexpression of EGFR-C, but not membranous EGFR, was correlated with poor prognosis of lung small cell carcinoma, oral small cell carcinoma, and thyroid cancer. To our knowledge, this study is the first to evaluate the prognostic effect according to the location of EGFR in CCAs.[35-37]

Downregulation of EGFR by endocytosis and lysosomal degradation has been regarded a key mechanism of signal attenuation.[38] Therefore, impaired downregulation of signaling receptors is strongly associated with carcinogenesis by leading to increased and uncontrolled receptor signaling.[38,39] Based on our results, overexpressed EGFR-M due to impaired downregulation might adversely affect prognosis of CCA. However, further studies are needed to confirm clinical significance of EGFR location in prognosis of CCA.

Distant organ metastasis is associated with poor prognosis in multiple cancers. Therefore, we investigated the correlation between distant metastasis and immunoassays with CCA TMA. After therapeutic resection for CCA, positive expression of IL-6 was significantly higher in patients with distant organ metastasis than in patients with locoregional recurrence. Positive expression rates of IL-6 in patients with distant organ metastasis and those with locoregional recurrence were 58% and 31%, respectively (p=0.010).

EMT is a structural and functional transformation of epithelial cells into mesenchymal cells. It is a key step of metastasis that is required for tumor cell migration and invasion from the primary tumor.[13,40] Previous studies have found that IL-6 is elevated in patients with CCA.[41,42] Yamada *et al.*[12] have reported that IL-6 can trigger EMT in CCA by promoting downregulation of epithelial cell markers and upregulation of mesenchymal marker. Like previous studies, our study also demonstrated that expression of IL-6 affected distant organ metastasis. On the other hand, E-cadherin (epithelial marker) and Snail (mesenchymal marker) known to be involved in the mechanism of EMT did not show a significant correlation with clinical prognosis or distant organ metastasis in this study.

TMA can obtain multiple cores from whole specimen and perform IHC staining through this, which has the advantage of being cost effective, time saving, and preserving tissues necessary for other studies or diagnoses.[43,44] However, it can be seen as a limitation that the cores obtained through this way may not be able to fully reflect the full specification.[45] In the previous studies, obtaining a core size of over 1 mm[46] or two or more cores[43,45,47-49] can increase the similarity as the result of obtaining through whole specimen, and in this study, two cores with a size of 2 mm were conducted to reduce this limitation.

Although previous studies have individually studied CCA and each IHC marker, few have comprehensively evaluated them. In this study, four IHC markers were analyzed alone and in combination (Supplementary Fig. 1). Snail or IL-6 was not a significant prognostic marker for OS (p=0.080 and p=0.363, respectively), but their combination was significantly related to OS (p=0.025). In addition, EGFR-C expression alone had no significance for OS (p=0.382), but its combination with IL-6 expression showed statistical significance for OS (p=0.027). This significant predictive value of the combined IHC markers has not been confirmed before, and further studies need to consider several combinations of IHC markers with survival and response to treatment.

In summary, overexpression of EGFR in membrane is significantly associated with shorter OS and DFS in surgically resected bile duct cancer patients. In addition, higher expression of IL-6 is a predictive marker for recurrence with distant organ metastasis after surgical resection in bile duct cancer patients.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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AUTHOR CONTRIBUTIONS

Study concept and design: D.W.K., J.K.L., J.K.P. Data acquisition: J.S.H., I.W.H., K.H.L., K.T.L., J.K.L., K.T.J., J.K.P. Data analysis and interpretation: D.W.K., S.H.S., Y.K., S.M.P. Drafting of the manuscript: D.W.K., Y.K., S.M.P., S.H.S., H.H.C. Critical revision of the manuscript for important intellectual content: H.H.C., S.H.S., H.K., J.K.P., K.T.J. Statistical analysis: S.H.S., H.H.C., H.K., J.K.P. Obtained funding: J.K.L., J.K.P. Administrative, technical, or material support; study supervision: J.K.P., J.K.L. Approval of final manuscript: all authors.

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SUPPLEMENTARY MATERIALS

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